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Down-Regulation of Microrna-432 in Cervical Mucus as a Pioneer Marker in Early Detection of Cervical Cancer

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Abstract

Objectives:-cancer of the cervix is one of the most common cancers and a leading cause of death in women worldwide .MicroRNAs (miRNAs), non-coding RNAs of 19–25 nucleotides in length,gene expression modulation partially by pairing with the 3′ untranslated region of their messenger RNAs target.

Aim of the study:to evaluate whether the detection of miRNA-432 is a biomarker for cervical cancer and the own precursor lesions by using cervical mucus and fresh punch biopsy specimens obtained from enrolled patients by using Real -Time PCR .

Materials and Methods:- Real-Time PCR was done to demonstrate the gene expression of microRNA-432 in cervical mucus and fresh punch tissue sample of patients with cervical cancer, precancerous , and control groups. The levels of expression of microRNA-432 were estimatedrelative to RNA messenger of Glyceraldehyde 3-phosphate dehydrogenase .

Results:-MicroRNA-432 expression level in tissue and mucus sample was significantly down-regulated in cervicalcancer in comparison to normal control,CINII and CIN III .MicroRNA-432 expression level in tissue and mucus sample was significantly down-regulated in CINII and CIN III in comparison to normal control .

Conclusion:-Cervical mucus- microRNA-432 gene expression can serve as less minimally-invasive biomarkers and standard screening tool for early diagnosis of precancerous lesion andcancer of the cervix.

Keyword:- MicroRNA-432, RT-PCR, cervical mucus and fresh punch tissue sample, cancer of the cervix, precancerous lesion .

Introduction

Cancer of the cervix is one of a common cancer in females, with an estimated 528,000 new cases and 265,000 deaths over the world in 2012 [(1)], and nearly 90% of cancer of the cervix mortality and occur in poor countries. Long time infection with a high-risk human papillomavirus (HPV), is an important cause of cervical cancer growing.Although, the fact that programmed cytology screening contributed to decreasing the cervical cancer incidence in under developed countries, cytology perseis not an ideal tool for screening due to its lesser sensitivity for detection of high-grade cervical intraepithelial neoplasia (CIN) lesions [(2)] and carcinoma [(3)] .Currently, HPV DNA tests have been inserted into the screening system program due to its high sensitivity compared to cytology. Nevertheless, their specificity is lesser because most HPV infections are temporary and do not manifested clinically as cervical lesions.That is why, a more biomarker specific needs to be developed[(2,4)]. Non-coding RNAs of 19–25 nucleotides in length,MicroRNAs (miRNAs) modulate gene expression by partially pairing with the 3′ untranslated region of their target messenger RNAs[(5)], and about 2/3 of human messenger RNAs are expected to be regulated by miRNAs.The miRNAs are impaired not only in tissues, but are also secreted into body fluids likeurine,serum,saliva,semen and vaginal fluid,We propose that mucus of the cervix was the bestmaterial for profiling neoplasia of the cervix[(6)] . MiRNA-432 dysregulation has been seem to be entangled in the carcinogenesis and progression of several typesof cancer, involving cervical and ovarian.[(7,8)] .

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Martial and method

Selected cases:-our study achieved for the period from March 2018 to January 2020. This is a prospective study, a total of 60 cases where by 50 patients with newly discoveredcervical cancer and precancerous cervicalcancer and 10 cases of healthy women ,All the study subjects were aged from 35 to 45 years, were recruited at the Gynecologicaland Obstetrics Department/ Teaching Hospital in DiawaniaCity. FNA cytology was adopted as a the standard diagnostic protocol for patients with cervical lesion in Pathology Department/ Teaching Hospital in Diawania City.The tissue diagnosis of CIN was based on the Richart classification. The results were interpreted using the Bethesda 2001 system.According to histopathological examinationcan classify the cervical lesion in to CINI (n = 10), CINII (n = 10), CINIII (n = 10), and squamous cell carcinoma (SCC), (n = 20) .in order to have miRNAs as a biomarker for cancer of the cervix and its precursor lesions, it is vital to select appropriate specimens not only from cervical cancer patient but also from female without cancer and healthy.extra amount of cervical mucus or vaginal discharge that disturb with the interpretation of cytology should be cleaned by a cotton swab and thrown before taking exfoliated cells for cytology. We regarded the ideal specimen those discarded materials .A mixture of circulating cells and local cellular cells such as secreta from cervical tissues, vaginal discharge, menstrual blood, and cervical exfoliated cells are the content of the cervical mucus. So, miRNAs in the cervical mucus surely be secreted from any of the origins above mentioned [(9)] .Mucus sample and punch biopsy were collected from the patients with cervical cancer and precancerous lesion (n=50) and collect mucus sample from healthy women (n=10). After punch biopsy of the cervical lesion , take fifty-pairs of fresh tissues from same cases of cervical lesion and normal adjacent tissues (NATs) which consider as internal control and preserved in Diethylpyrocarbonate (DEPC) water for total RNA extraction and for RT-PCR. Another 50 pairs specimens of both cervical lesion and normal adjacent tissues for histopathological examination.Now thecommonly used staging system for cancer of the uterine cervix worldwide is the International Federation of Gynecology and Obstetrics (FIGO) stage [(9)].

MiRNA isolationfrom tissue and mucus: mucus samples were compiled between 8:00 and 9:00 a.m. following centrifugation for 30 min at 2,650 g, and samples were stored at 80o c. Tissue samples were homogenized in adenaturing lysis solution and dissolved RNA was stored at -20°C before use. RNA was extracted from mucus and fresh tissues using the Trizol reagent (Bioneer, Korea) based on the manufactures instructions. RNA quality was studied with a NanoDrop 1000 spectrophotometer.

Real-time RT-PCR for miR-432 quantification: The miR-432 was studied using the TaqManmiR RT kit protocol (Applied Biosystems, Foster City, CA, USA) including in a the step one of RT with an miR-specific primer (used miR-base ,as a database to design the primers) and in step two the real-time PCR with TaqMan probes. Transcriptase reverse reactions were done to produce cDNAs in a volume of fifteen ml using ten ng total RNA for each sample, 50 nM stem–loop RT primer, 1! RT buffer, 1 mM each of dNTPs, 3.33 U/ml Multi- Scribe transcriptase reversed, and 0.25 U/ml RNase inhibitor. After incubation on ice for 5 min, then the program of heating started with 30 min at 16 8C, 30 min at 42 8C, 5 min at 85 8C, and hold at 4 8C. Real-time PCR wasadopted in triplicate in a 96-well optical plate on the Applied Biosystems 7700 Sequence Detection System.each sample a volume of 20 ml included! TaqMan Universal PCR Master Mix, 1 ml specific miR Assay Mix (Applied Biosystems), and 1.34 ml RT product. The responses were incubated at 50 8C for 2 min and 95 8C for 10 min, followed by 40 cycles of 95 8C for fifteen s and 60 8C for one min. All miR-432 quantification data were normalized to housekeeping gene like Glycer aldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA of GAPDH gene primers and probe were designed by using NCBI-Gene Bank data base and Primer 3 plus design online. The primers were performed as follows: miR-432 forward, 5'-AAC GAG ACG ACG ACA GAC T-3' and reverse, 5'-CTT GGA GTA GGT CAT TGG GT .ThecDNAs primer of GAPDH as design as Random Hexamer primer and the primer used in qPCR was: forward, CAGCCGCATCT-TCTTTTGC and reverse, TTAAAAGCAGCCCTGGTGAC. Taq-Man probe for mGAPDH was: FAM-CCAGCCGAGCCACATCGCTC-TAMRA. The data obtained of RT-qPCR for miR-

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432 and GAPDH were analyzed by the relative quantification expression of gene levels (fold change) were based on the Ct values by using the Livak method (Fold change = 2-ΔΔCT) that described by (Livak and Schmittgen)[10] .

Statistical analysis: Microsoft Office Excel 2007 andSPSS version were adopted in data analysis, Chi-square test and Fisher exact test were adopted to study association between any double nominal variables. (P-value) of less than or equal to 0.05 was accepted as significant.

Results

1- the tissue miRNA-432 gene expression of squamous cell carcinoma and precancerous cervical lesion were compared with normal adjacent tissues.

Mean cancer tissue of miR-432 was statistical significantly lower from that NATs, -20 ± 0.1 versus, 1± 0.1 respectively (P<0.002). Mean fold change of miR-432 in the tissue sample of CINI was un-change in comparison to that NATs and no statistically significant difference from that NATs (P> 0.05). Mean cancer tissue of miR-432 was statistical significantly lower than from CINI tissues (P<0.001).

Mean fold change level of miR-432 in the tissue sample of CIN II and CIN III was decreased in comparison to that NATs and statistically significant difference from that NATs (P <0.05) .Mean fold change level of miR-432 in the tissue sample of CIN III was higher decreased in comparison to CIN II and statistically significant difference (P <0.05).Mean cancer tissue of miR-432 was statistical significantly lower from that CINII and CIN III tissues (P<0.001).As shown in the table (1).

Table (1): the tissue level comparisonof miRNA-432 gene expression betweensquamous cell carcinoma , precancerous and normal adjacent tissues .

Groups	*N*	*Mean*	*SD*
NATs	50	1	±0. 1
SCC	20	-20	±0.1
CIN I	10	1	±0.1
CIN II	10	-7	±0.1
CIN III	10	-15	±0.1

2-Comparison the cervical mucus miRNA-432 of squamous cell carcinoma and precancerous lesion with apparently healthy controls

Mean cancer of the cervical mucus miR-432 was statistical significantly lower from those apparently healthy controls, (-18 ± 1.5) versus, (1± 0.01), respectively (P<0.001). Mean fold change of miR-432 in the cervical mucus of CIN I was un-change in comparison tothose apparently healthy controls and no statistically significantly different fromthose apparently healthy controls (P> 0.05). Fold change of miR-432 in mucous of SCC was statistical significantly lower from mucus of CIN I (P<0.001). Mean fold change level of miR-432 in the mucus sample of CIN II and CIN III was decreased in comparison to those apparently healthy controls and statistically significant difference fromthose apparently healthy controls (P <0.05) .Mean fold change level of miR-432 in the mucous sample of CIN III was higher decreased in comparison to CIN II and statistically significant difference (P <0.02). Mean cancer mucus of miR-432 was statistical significantly lower from mucus of CINII and CIN III (P<0.001). seen in the table (2).

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Table (2): Comparison of the mucus fold change of miRNA-432 gene expression betweensquamous cell carcinoma , precancerous , and control groups .

Groups	*N*	*Mean*	*SD*
Control	10	1	±0.41
SCC	20	-18	±1.5
CIN I	10	1	±0.01
CIN II	10	-5	±0.01
CIN III	10	-13	±0.01

3-Correlation between the age of the patient with the fold change of miRNA-432.

The results of present study presented that there was no significant association between fold change miR-432 of SCC ,CIN I,CIN II ,CIN III and age of patients in those of ≤45 years and>45 years, (P>0.005) .

4-Correlation between the tumor size with the fold change of miRNA-432 .

The results of present study presented that there was no significant association between fold change miR-432 of SCC and tumor size (P>0.005).

5-Predictive value of miRNA-432 in the mucus and tissue of patients .

To evaluate the diagnostic value ofmucus and tissuemiR- 432 for SCC ,CIN II and CIN III by using the RT-qPCR technique, a Receiver Operator Characteristic (ROC) curve analysis was done:

A-Comparison of the tissue miRNA-432 of squamous cell carcinoma and precancerous versus that of normal adjacent tissue .

The best cutoff value for tissue miR-432 in SCC in comparison to that of NAT was (-17 fold change) with a specificity of 100%, a sensitivity of 85% and accuracy(AUC) was excellent. The best cutoff value for tissue miR-432 in CINII and CINIII was (-7 fold change and -15fold change) respectively with accuracy excellent .As shown in the table (3) .

Table (3): -The cut of the value of tissue miRNA-432 in the differentiation of patients withsquamous cell carcinoma , precancerous from that of normal adjacent tissues .

Parameter	*Cutoff value*	*Sensitivity*	*Specificity*	*Accuracy (AUC)*	*P-value*
Tissue miR-432 of SCC	-17 fold change	85 %	100%	% (0.90)	<0.001
Tissue miR-432 of CIN II	-7 fold change	80 %	100%	% (0.90)	<0.001
Tissue miR-432 of CIN III	-15 fold change	90 %	100%	% (0.90)	<0.001

B-Comparison of the mucus miRNA-432 of squamous cell carcinoma and precancerous versus healthy control group.

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The best cutoff value for mucus miR-432of SCC in comparison to those apparently healthy control groups was (-15 fold change) with a specificity of 100%, a sensitivity of 85% and accuracy(AUC) was excellent. The best cutoff value for mucus miR-432 of CINII and CINIII was (-3fold change and -10fold change) respectively with excellent accuracy .As shown in the table (4) .

Table (4): -The cut of the value of mucus miRNA-432 in the differentiation of patients withsquamous cell carcinoma ,precancerous from healthy control groups

Parameter	*Cutoff value*	*Sensitivity*	*Specificity*	*Accuracy (AUC)*	*P-value*
Mucus miR-432 of SCC	-15 fold change	85 %	100%	% (0.90)	<0.001
Mucus miR-432 of CIN II	-3 fold change	80 %	100%	% (0.90)	<0.001
Mucus miR-432 of CIN III	-10 fold change	90 %	100%	% (0.90)	<0.001

Discussion

For studying miRNAs as a biomarker, it is serious to use a less invasive method in obtaining a specimen. Excess amount of cervical mucus or vaginal discharge obscuring the interpretation of cytology might be cleaned by a cotton swab and discarded before taking exfoliated cells for cytology. We adopted these discarded materials as an ideal specimen. a mixture of circulating cells and local cellular cells such as secreta from cervical tissues, vaginal discharge, menstrual blood, and cervical exfoliated cells all these were the content of cervical mucus. So, miRNAs in the mucus of the cervix might be secreted from any of the origins mentioned above [(11)] .The aim of this study was to select whether down-regulated miRNA-432 from profiling of mucus could determine the high-grade CINs and cancer.our results revealed that miR-432 was expressed at low steps in high-grade CINs and cancer of the cervix in the cervical mucus as well as in tissues from the patient oneself .our results suggest that down-regulation of miR-432 may be included in the progression and development of cervical cancer.Our results was agreement with other study like study of shanzongwang*et al*[(12)].

The best cutoff value for tissue miR-432 in SCC in comparison to that of NAT was (-17 fold change) with a specificity of 100%, a sensitivity of 85% and accuracy(AUC) was excellent ,proposing that this assay might be a good biomarker for diagnosing cervical cancer.The best cutoff value for tissue miR-432 in CINII and CINIII was (-7 fold change and -15fold change) respectively with accuracy excellent .These results suggesting that miR-432 could be a potential biomarker for identifying high-grade CINs.The best cutoff value for mucus miR-432 of SCC in comparison to those apparently healthy control groups was (-15 fold change) with a specificity of 100%, a sensitivity of 85% and accuracy(AUC) was excellent and the best cutoff value for mucus miR-432 of CINII and CINIII was (-3fold change and -10fold change) respectively with excellent accuracy.These results suggesting that mucus miR-432 could bepromising biomarker and screening test for early diagnosis of high grade precancerous and cervical cancer .

Conclusion: Cervical mucus microRNA-432 gene expression can serve as less minimally-invasive biomarkers and standard screening tool for early detection of precancerous lesion from that normal healthy control and from that squamous cell carcinoma .Nevertheless , there were some restrictions to this study,the influence of miR-432 on the cell cycle of the cancer of the cervix was not scrutinized and the interpretation of the test in

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terms of precision should be parallel with cytology or HPV test results taking from population-based screening. The sample size of the present study was small ,stage of squamous cell carcinoma

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